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Imazethapyr Inhibition of Acetolactate Synthase in *Rhizobium* and Its Symbiosis with Pea

Mercedes Royuela,[1]* Azucena Gonzalez,[1] Cesar Arrese-Igor,[1] Pedro M. Aparicio-Tejo[1] & Carmen Gonzalez-Murua[2]

[1] Dpto Ciencias del Medio Natural, Univ. Pública de Navarra, Campus Arrosadía, E-31006 Pamplona, Spain
[2] Dpto Biología Vegetal y Ecología, Univ. del País Vasco, Apdo 644, E-48080 Bilbao, Spain

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Abstract: Acetolactate synthase (ALS) activity extracted from *Rhizobium leguminosarum* biovar. *viciae* has been characterized. The optimum pH for extraction was 7·6 and for the assay 7·0. The K_m for pyruvate was 7·2 mM, and the enzyme was saturated at 40 mM. An obligatory requirement of TPP and Mg^{2+} for full ALS activity was observed. Valine was the only branched-chain amino acid that caused ALS feedback inhibition. The specific activity of *Rhizobium* ALS was nearly 20 times the activity found in pea (*Pisum sativum*) leaves. Bacteroids from pea nodules also showed high ALS activity, and the nodule plant fraction had higher ALS activity than other plant tissues. ALS sensitivity to imazethapyr was also dependent on the source: ALS activity of free-living *Rhizobium* and bacteroids was slightly more tolerant than that of other pea tissues, but the differences were less than those found in rates of specific activity. It is proposed that the high ALS activity expressed by *Rhizobium*, both as free-living bacteria and as bacteroids, is related to the growth tolerance of rhizobia to imazethapyr and is also related to the relative tolerance of symbiotic pea plants. © 1998 SCI

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1 INTRODUCTION

Acetolactate synthase (ALS; EC 4.1.3.18, also referred to as acetohydroxyacid synthase; AHAS) catalyzes the first common step in the biosynthesis of branched-chain amino acids: valine, leucine and isoleucine. This metabolic pathway plays an important role in plants and micro-organisms but is absent in mammals, which must obtain these amino acids in their diet. There is strong interest in this enzyme, as it has been shown to be the target of at least three structurally unrelated classes of modern and very potent herbicides, sulfonylureas, imidazolinones and triazolopyrimidines.[1,2] In turn, these herbicides may provide new tools for characterizing the enzymes of the branched-chain amino acid biosynthetic pathway and the pathway itself.

Over the past decade, ALS-inhibiting herbicides have become extensively used world-wide, mainly for selective weed control in a variety of crops. However, the inherent tolerance to these herbicides is not based on ALS insensitivity in the tolerant crop. Instead, natural selectivity of sulfonylurea and imidazolinone herbicides is attributed to the rapid metabolic inactivation of the herbicide in tolerant crops.[3,4] ALS from all plant species so far examined is highly sensitive to sulfonylureas[5,6] and imidazolinones[7] regardless of the plant's tolerance.

The application of herbicides in agricultural systems may exert side effects on the soil microflora, including a possible shift in microbial community structure.[8,9] This may be particularly true in the case of compounds

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which interfere with amino acid biosynthesis and, therefore, are also potentially effective on microbial metabolism. This possibility was investigated for the environmentally safe broad-spectrum herbicide glyphosate.[10] Although the possible effects of glyphosate on microbial community structure are thought to be negligible, a differential sensitivity of the herbicide target has been demonstrated in free-living bacteria.[11] With ALS-inhibitor herbicides, Forlani *et al.*[12] showed that the differential tolerance of some rhizobacteria to sulfonylureas and imidazolinones was related to a differential sensitivity of ALS, the target enzyme.

In the Enterobacteria (*Escherichia coli* (Mig.) Cast. & Chalm and *Salmonella typhimurium* Cast. & Chalm) in which ALS have been extensively studied, there are three different forms of the enzyme. Each one has a different mode of regulation at the level of gene expression and/or feedback inhibition by the final products of the pathway and shows differential sensitivities to these herbicides.[2,13] However, very little is known about the properties of ALS activity and whether it occurs as a single enzyme or as several isozymes in other bacterial sources. In plants, two isozymes have been described in corn[14] and *Brassica napus* L.,[15] but only a single enzyme in pea,[16] barley[17] and wheat.[18]

Because of their role in biological nitrogen fixation in association with legumes, the Rhizobiaceae are one of the most important agronomically related bacterial groups. In a previous work,[19] growth of *Rhizobium leguminosarum* biovar. *viciae* was shown to be tolerant to imazethapyr (a member of the imidazolinone class). This herbicide did not directly affect *Rhizobium*, and doses 700 times higher than the recommended field application rate were required to cause minor effects on *Rhizobium* growth in a defined medium. Also, the nodulation ability of *Rhizobium* treated with imazethapyr was not affected. Differences in sensitivity to imazethapyr of 1000-fold were observed between pea (*Pisum sativum* L.) plants and *Rhizobium*, measured as growth parameters after herbicide treatment. It has also been shown that other *Rhizobium* species seem to be tolerant to high rates of another ALS inhibitor: the sulfonylurea chlorsulfuron.[20–22] The biochemical basis of such a tolerance has not been elucidated.

The establishment of an effective *Rhizobium*–legume symbiosis requires the differentiation of free-living rhizobia into bacteroids, which display important physiological differences compared with free-living rhizobia, including nitrogen fixation capability and amino acid metabolism.[23] Nodulated pea plants may show different responses to environmental stresses compared to non-symbiotic plants, and this fact may also affect their responses to xenobiotics. Thus, a higher tolerance to imazethapyr by nodulated pea has been described[19] and this may be related to differences in the herbicide target.

The aim of this work was to investigate ALS, the imazethapyr target, in both *Rhizobium* and nodulated pea plants. The work was focused on the optimization of the extraction procedure and assay for *Rhizobium* ALS. Also some properties of ALS are shown. ALS activity was determined in different organs of nodulated peas: leaves, roots, nodule plant fraction and bacteroids and the dependence of the ALS response to imazethapyr on the nitrogen source was investigated. The results are discussed in relation to tolerance of free-living *Rhizobium* to imazethapyr and the better performance of nodulated pea plants compared to non-nodulated pea in the presence of this herbicide.

2 MATERIALS AND METHODS

2.1 Bacterial culture

R. leguminosarum biovar. *viciae* strain NLV8 was grown, under sterile conditions, in pure culture in a complex medium[24] (YMB). Eighty millilitres of this medium, sterilized at pH 6·8, were prepared in 250-ml Erlenmeyer flasks and cultures were incubated at 26°C on a rotary shaker at 200 rpm. Bacterial growth was determined by optical density measurements at 680 nm.

2.2 Plant growth

Seeds of *Pisum sativum* L. cv. Frilene were surface sterilized as described by Vincent[24] and germinated in Petri dishes at 26°C in the dark for two days. Germinated seeds were planted in 2-litre pots with vermiculite as the substrate. Seedlings were inoculated with 1 ml of the bacterial suspension (*c.*10^8 cells) grown in YMB. Plants were watered with a nitrogen-free nutrient solution[25] three times a week. Non-nodulated plants received the same nutrient solution supplemented with potassium nitrate (20 mM). Seedlings were grown in growth chambers with a photon flux density of 400 μmol m^{-2} s^{-1}, 16 : 8 h light : dark and 26 : 18°C (day/night). Plants were harvested four weeks after planting and plant parts were separated (leaves, roots and nodules), frozen in liquid nitrogen and stored at −80°C until use.

2.3 Extraction of bacterial ALS

Exponentially growing *R. leguminosarum* cells (*c.*10^8 bacteria ml^{-1}) were harvested by centrifugation at 12 000*g* for 10 min and washed twice in potassium phosphate buffer (0·1 M; pH 7·6). The cell paste was suspended in potassium phosphate buffer (0·1 M; pH 7·6), flavin adenine dinucleotide (FAD; 0·1 mM), dithiothreitol (0·5 mM), ethylenediaminetetraacetic acid (EDTA; 10 mM) and glycerol (200 ml $litre^{-1}$) and then lysed by ultrasonic disruption at 14 μm and 0°C using 10-s exposures with 10-s interruptions over an 8-min

period with a Soniprep 150 (MSE). Unbroken cells and debris were removed by centrifugation at 35 000*g* for 30 min at 4°C. The supernatant was desalted on a Sephadex G-25 column equilibrated with the same buffer. The desalted enzyme was used immediately for assays.

2.4 Extraction of plant ALS

Leaves (2 g FW) or roots (2·5 g FW) were homogenized in a mortar with the extraction buffer described by Muhitch:[26] potassium phosphate buffer (50 mM; pH 7·5) containing sodium pyruvate (5 mM), $MgCl_2$ (5 mM), EDTA (5 mM), FAD (100 μM), thiamine pyrophosphate (TPP; 1 mM) and glycerol (100 ml litre^{-1}). Polyvinylpolypyrrolidone (20 g litre^{-1}) was added to the leaf extraction buffer as described by Ray.[5] The homogenate was filtered through four layers of cheese cloth and centrifuged at 20 000*g* for 20 min. The supernatant was fractionated by ammonium sulfate (65%) and allowed to stand, with stirring, for 45 min on ice. It was then centrifuged at 20 000*g* for 20 min and the supernatant discarded. The precipitate was resuspended in the same extraction buffer without sodium pyruvate and then desalted on a Sephadex G-25 column equilibrated with the same buffer. The extract was used immediately for assays.

2.5 Extraction of nodule ALS

Nodules (0·5 g FW) were homogenized in a mortar and pestle with the same buffer as described above. The homogenate was centrifuged at 500*g* for 2 min to discard nodule debris. To separate nodule plant fraction from bacteroids, the resultant supernatant was again centrifuged at 12 000*g* for 10 min. Each fraction was washed twice to ensure that it was free of cross-contamination. ALS from the nodule plant fraction (supernatant) was extracted as for leaves and roots, and ALS from bacteroids (pellet) as from free-living *Rhizobium*.

2.6 ALS assay

ALS activity was measured by estimation of the product, acetolactate, after conversion by decarboxylation to acetoin in the presence of acid. For the bacterial assay, aliquots were added to the reaction mixture in a total volume of 0·5 ml, consisting of potassium phosphate buffer (0·1 M; pH 7·0) containing sodium pyruvate (40 mM), $MgCl_2$ (10 mM), TPP (0·1 mM) and FAD (25 μM). The same reaction mixture was used for the plant and nodule plant fraction except that TPP was increased to 0·62 mM and FAD to 34 μM. The amount of protein added to the reaction mixture ranged from 30 μg for *Rhizobium* and bacteroid assays to 140–200 μg for plant fractions.

The reaction mixture was incubated at 37°C for 1 h, then the reaction was stopped by adding H_2SO_4 (3 M; 50 μl). The reaction product was decarboxylated at 60°C for 15 min and the amount of acetoin formed was determined by the Westerfeld method[27] by adding creatine (5 g litre^{-1}; 0·5 ml) and α-naphthol (50 g litre^{-1}, in 2·5 M NaOH) to each tube followed by incubation at 60°C for 15 min. The optical density was then read at 525 nm. Appropriate checks for acetoin not derived from acetolactate were made by stopping the assay with 4 M sodium hydroxide. This was estimated in all assays and subtracted from the overall acetoin production to give actual ALS activities. Protein concentration was determined according to Bradford.[28]

Herbicide and amino acids solutions used to test the ALS sensitivity were prepared in potassium phosphate buffer (50 mM; pH 7·0) and stored at 4°C until use. For inhibition studies, different amounts of imazethapyr and amino acids were added to the reaction medium. Technical grade imazethapyr (98%) was used in all experiments.

Each assay was run at least in triplicate and the experiments were repeated at least three times.

3 RESULTS AND DISCUSSION

3.1 *Rhizobium* ALS characterization

It has previously been described that ALS from different sources shows different requirements and, therefore, an optimization of the pH, cofactors and substrate saturation for assaying ALS is recommended for each new species.[14] In order to find the optimum pH for *R. leguminosarum* ALS, the extraction and assay were performed under the method described above using several different pH values (6 to 8·5). The highest ALS activity was obtained when the extraction was carried out at pH 7·6 (Fig. 1A). Likewise, 7·0 was the optimum pH for the ALS reaction as shown in Fig. 1B. Therefore, *Rhizobium* ALS was extracted at pH 7·6 and 7·0 was used for the assay as a routine protocol. These values are in the range of pH reported for micro-organisms and were the same as those determined for *Escherichia coli*[29] and *Saccharomyces cerevisiae* Meyer ex Hansen.[30]

Figure 2 shows that the pyruvate saturation curve is hyperbolic and ALS activity follows Michaelis–Menten kinetics, with the calculated K_m for pyruvate being 7·2 mM and the activity was saturated at 40 mM, without any apparent substrate inhibition at higher substrate concentrations. Similar results have been reported for ALS from *Salmonella*[31] ($K_m = 8$) and *Corynebacterium*[32] ($K_m = 8{\cdot}3$). K_m values ranging from 1·5 to 5 mM have been reported for plants.[14,16,33] This

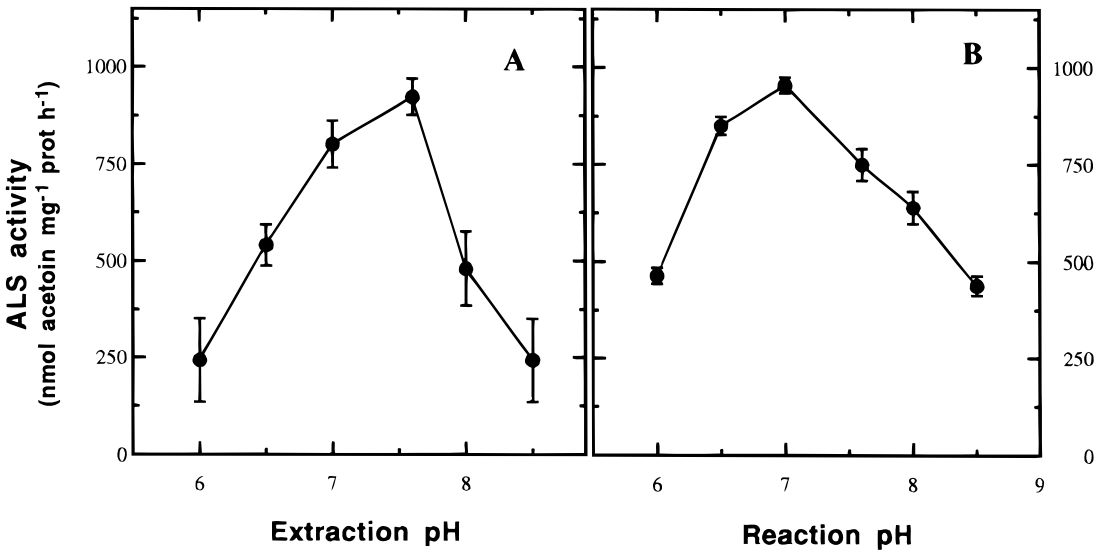

Fig. 1. Influence of (A) extraction pH and (B) reaction pH on ALS activity in *Rhizobium leguminosarum*. Vertical bars represent standard error of 12 replicates.

hyperbolic response towards the substrate together with the behaviour towards the inhibitor (imazethapyr, see Fig. 5) strongly suggests that *Rhizobium* has a single enzyme or, if several enzymes are present, they share the same K_m for pyruvate and K_i for imazethapyr.

Although the ALS requirements are not common for every organisms, the requirement for TPP, Mg^{2+} and FAD for full enzyme activity are widespread.[34] TPP and Mg^{2+} are known to be involved in the catalytic reaction of condensing pyruvate to acetolactate, whilst the function of FAD is not clear, since no net oxidation or reduction occurs in the reaction. Durner and Böger[17] showed that high FAD concentrations facilitate dissociation of the oligomeric species of barley ALS.

The study of ALS cofactors in *Rhizobium* and pea indicates an obligatory requirement of TPP and Mg^{2+} for full ALS activity (Table 1). Omission of TPP or Mg^{2+} produced a decrease in activity to 65% and 71%, respectively, of the control in *Rhizobium*, and to 65% and 60% in pea. TPP is essential in ALS activity for most organisms because its binding to ALS is necessary to produce pyruvate decarboxylation.[35] However, it has

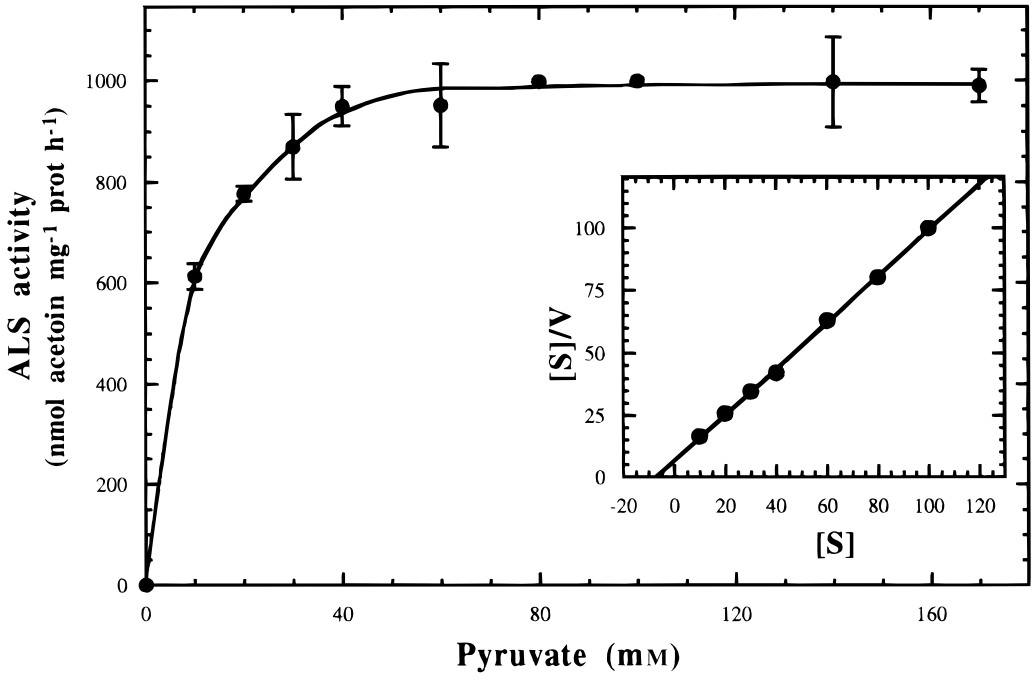

Fig. 2. Pyruvate saturation curve for *Rhizobium leguminosarum* ALS. The inset is a S/V versus S plot (Hanes Woolf plot) of the data. [S] is the substrate concentration (pyruvate, mM) and V is the enzymatic activity expressed as μmol acetoin mg^{-1} prot h^{-1}. Vertical bars represent standard error of nine replicates.

TABLE 1
Cofactor Requirements for ALS Activity in *Rhizobium leguminosarum* and Pea Leaves

Cofactors omitted from the reaction medium	*ALS activity (nmol acetoin mg^{-1} prot h^{-1}) ($\pm SE$)*[a]	
	Rhizobium	*Pea*
None (complete medium)	950 (±72)a	37·0 (±1·8)a
FAD	929 (±36)a	33·3 (±2·1)a
TPP	621 (±28)b	24·1 (±2·9)b
Mg^{2+}	679 (±16)b	22·2 (±2·3)b
TPP, Mg^{2+}	658 (±30)b	25·9 (±2·5)b
TPP, Mg^{2+}, FAD	672 (±37)b	26·1 (±2·9)b

[a] $n = 9$
Same letter in a column indicates that values are not significantly different at 5% level in Fisher's-protected LSD

been shown that ALS from *Euglena gracilis* Klebs and *S. cerevisiae* do not require exogenous TPP.[36] Mg^{2+} plays an important role in the binding of TPP to ALS.[14] Nevertheless, there are some organisms whose ALS shows highest activity in the absence of Mg^{2+}.[36–38] When TPP and Mg^{2+} were both omitted from the reaction medium, ALS activity of *Rhizobium* and pea exhibited no further inhibition than each one separately. Moreover, Mg^{2+} was only effective when TPP was present in the reaction medium. This behaviour was similar to that reported for *Neurospora crassa* Shear & Dodge ALS.[39]

Our results show that a FAD requirement for *Rhizobium* and pea ALS is not clear, since its omission from the reaction medium did not affect the ALS activity (Table 1). However, we cannot discard the possibility that this cofactor is necessary at low concentrations, as residual FAD may be bound to ALS because of its presence in the extraction buffer. The concentration used in the buffer may be sufficient to stabilize activity due to a tight union to ALS.[31] Thus, we cannot discard that FAD may be essential for *Rhizobium* and pea ALS at low concentrations.

3.2 Feedback inhibition by branched-chain amino acids

A feedback inhibition of ALS activity by branched-chain amino acids has been described in different organisms.[40] Nevertheless, the extent of such a regulation shows great variability. In our work, ALS activity from *Rhizobium* showed a different response to each branched-chain amino acid (Fig. 3), with a lack of inhibition by leucine and isoleucine. Indeed, increasing leucine concentrations enhanced ALS activity. Only valine caused inhibition of ALS activity: 1 mM valine inhibited the *Rhizobium* ALS activity by *c.*13%, with only a slight increase in inhibition being observed at a higher concentration (10 mM). Similar responses to valine have been observed in *E. coli*,[29] *Chlorella emersonii*,[41] and *Serratia marcescens* Bigio.[42] Also, insensitivity to valine has been described in *Streptococcus bovis*.[38]

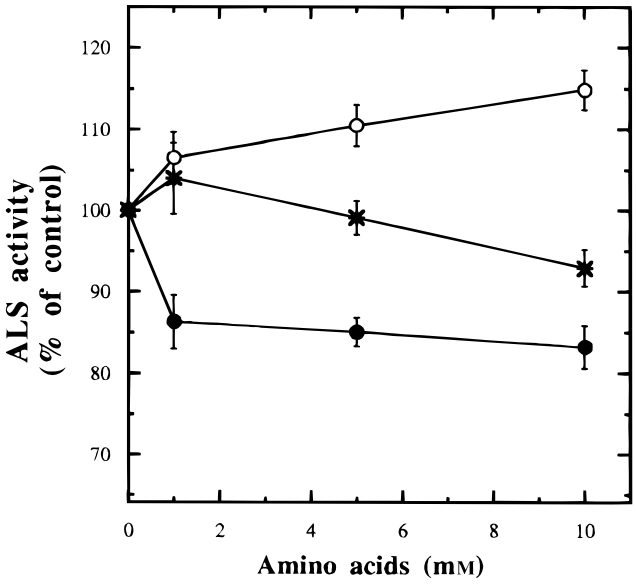

Fig. 3. Influence of branched-chain amino acids on the ALS activity of *Rhizobium leguminosarum*. (●) Valine, (○) leucine and (∗) isoleucine. Vertical bars represent standard error of nine replicates.

3.3 ALS activity in nodulated pea plants

ALS occupies the same central role in valine, leucine and isoleucine biosynthesis in plants as it does in microorganisms. ALS occurs in small quantities in plant tissues and is located in plastids,[43] with total and specific activities ranging widely among different species.[40] We found ALS specific activities in pea leaves and roots ranging from 35 to 41 nmol acetoin mg^{-1} prot h^{-1}, similar to those described by Shimizu *et al.*[16] but lower than those described by Singh *et al.*[14] Also, it has been reported that different tissues in the same species may show different activities, activity being higher in embryos than in mature tissues.[6] In general, metabolically active tissues, such as meristems, show higher ALS specific activities and ALS transcript levels.[44]

Our results (Table 2) showed that ALS specific activity is evenly distributed in leaves and roots in nodulated pea. Also, its expression and distribution are not affected by the nitrogen source, as the activity was the same for nodulated and non-nodulated plants (35·86 versus 37·0, respectively in leaves, and 40·89 versus 39·26, respectively in roots). However, ALS specific activity was 3-fold higher in the plant fraction of nodules. In contrast, bacteroids showed an activity in the same range as free-living bacteria, although it was significantly lower (707 versus 949, respectively), which

TABLE 2
ALS Activity Extracted from Different Sources of Nodulated and Non-nodulated Pea Plants and from Free-Living *Rhizobium leguminosarum*

ALS source	*ALS activity (nmol acetoin mg^{-1} prot h^{-1}) ($\pm SE$)*[a]
Leaves of nodulated plants	35·86 (±2·22)a
Leaves of non-nodulated plants	37·01 (±1·81)a
Roots of nodulated plants	40·89 (±3·53)a
Roots of non-nodulated plants	39·26 (±3·09)a
Nodule plant fraction	109·87 (±15·77)b
Bacteroids from pea nodules	707·05 (±19·76)c
Free-living *Rhizobium*	949·50 (±72·10)d

[a] $n = 9$
Same letter in a column indicates that values are not significantly different at 5% level in Fisher's-protected LSD.

was 20 times greater than the activity found in plant tissues.

These results are of significance because legumes treated with ALS-inhibitor herbicides under field conditions are generally in symbiosis with *Rhizobium*. Their response, therefore, may be dependent on their ALS activity, as one of the factors that can contribute to a higher herbicide tolerance in overexpression of the target enzyme. In this sense, the high activity shown by bacteroids may lead to the nodulated plants having an additional source of ALS activity and, hence, of branched-chain amino acids. Although bacteroid contribution to the overall amino acids biosynthesis in nodules has not been comprehensively studied, it has been reported that some amino acids (alanine and aspartate) can efflux from isolated soybean bacteroids and isolated symbiosomes in long-term experiments.[45] Branched-chain amino acids frequently represent less than 10% of total free amino acids[6] and also *in-vivo* application of imazethapyr to nodulated pea plants does not produce a decrease in the branched-chain amino acids pool in nodules (Royuela, M., unpublished results). This is in contrast to the situation shown in other plant tissues in response to imidazolinones.[46,47] If nodules provide an effective source of branched-chain amino acids, this would explain why nodulated peas were less affected than nitrate-reducing plants by imazethapyr, as shown in a previous study.[19]

3.4 Imazethapyr inhibition of ALS

Studies on inhibition of enzyme activity are normally performed with purified enzymes. However, because of the lability of ALS this has proved to be difficult. In the few cases in which purified ALS and crude extracts have been compared the inhibitory effects of different compounds are identical.[16,33]

Inhibition of ALS from enteric bacteria and higher plants by imidazolinones has been reported to be time-dependent and biphasic,[2,48] indicating that the mode of action of these herbicides should be examined under identical conditions to allow direct comparisons. Analysis of the time-dependent inhibition by imazethapyr (Fig. 4) shows an increase to a steady state over the study period. The rate of conversion from initial binding to final binding depended on the concentration of imazethapyr, with more rapid changes occurring with higher inhibitor concentrations (data not shown). The inhibitory effect of imazethapyr was higher in pea leaves than in *Rhizobium*, but the time course of the inhibition was similar in both cases (Fig. 4) and was also similar to the pattern described for ALS inhibition by imazapyr in maize.[4] This was also true for ALS from non-nodulated peas (data not shown). Hawkes and Thomas[49] suggested that this apparent slow phase of ALS inhibition by herbicides may be due to an isomerization of the enzyme–inhibitor complex to a more tightly bound form, or, more probably, due to slow irreversible inactivation of ALS.

Despite the fact that *Rhizobium* growth was not affected by high imazethapyr concentrations,[19] this herbicide behaved as an inhibitor of ALS activity from *Rhizobium*, when added to the reaction medium (Fig. 5). ALS activity decreased with increasing imazethapyr concentrations, showing a 70% inhibition at 2 mM whilst 0·6 mM was necessary to obtain a 50% inhibition (I_{50}). Thus, *Rhizobium* ALS is slightly more tolerant to

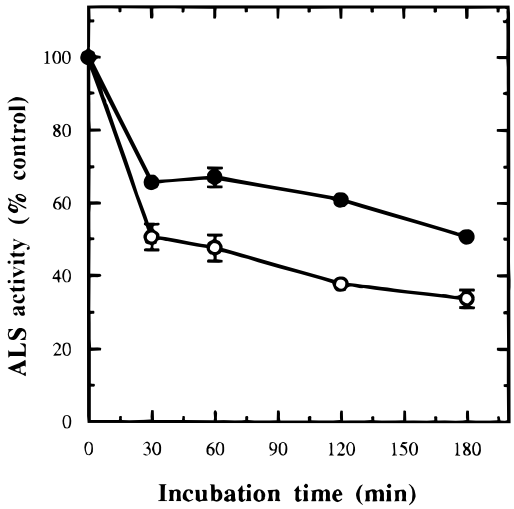

Fig. 4. Time-course of the imazethapyr (0·1 mM) effect on ALS activity in *Rhizobium leguminosarum* (dark symbols) and pea leaves (white symbols). The rate of the reaction in the control experiments decreased over the time-course shown, and the data are expressed as a percentage of the corresponding uninhibited controls. Vertical bars represent standard error of nine replicates.

TABLE 3
Effect of 0·1 mM Imazethapyr on ALS Activity Extracted from Different Sources of Nodulated and Non-nodulated Pea Plants and from Free-Living *Rhizobium leguminosarum*

ALS source	*ALS activity with 0·1 mM imazethapyr*	
	nmol acetoin mg^{-1} *prot* h^{-1} $(\pm SE)^a$	*% of control*
Leaves of nodulated plants	17·02 (±1·25)	47·5ab
Leaves of non-nodulated plants	17·14 (±1·07)	46·3a
Roots of nodulated plants	18·85 (±0·36)	46·1a
Roots of non-nodulated plants	19·67 (±0·59)	50·1ab
Nodule plant fraction	59·11 (±2·31)	53·8b
Bacteroids from pea nodules	472·95 (±28·3)	66·8c
Free-living *Rhizobium*	637·25 (±24·7)	67·1c

[a] $n = 9$
Same letter in a column indicates that values are not significantly different at 5% level in Fisher's-protected LSD.

imazethapyr than pea ALS, whose I_{50} (in our experimental conditions) was 0·1 mM (Fig. 5), a similar value to that previously reported (0·083 mM).[7]

However, the lack of response to imazethapyr by *Rhizobium* growth does not seem to be related to this higher *in-vitro* I_{50}, since tolerance based on a different target sensitivity commonly requires I_{50} differences of several orders of magnitude.[50,51] Indeed, whereas differences in ALS I_{50} allowed for an explanation of a differential tolerance among rhizosphere bacterial species to ALS-inhibitor herbicides,[12] there is no apparent relationship between ALS I_{50} and the corresponding plant sensitivity to these herbicides.[40]

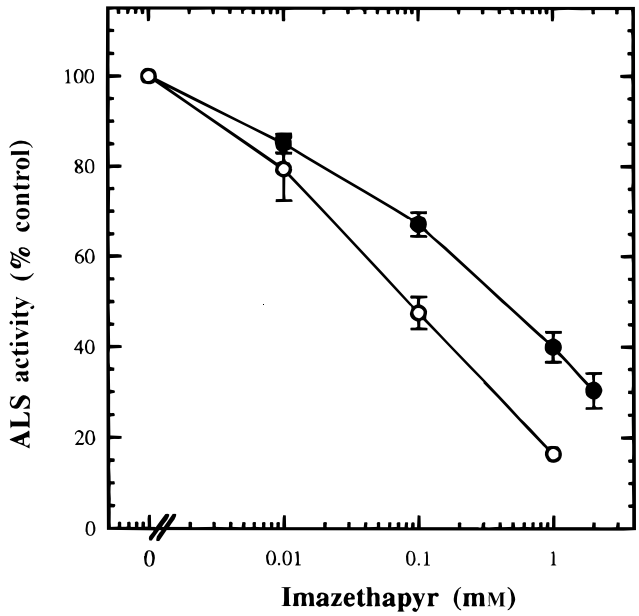

Fig. 5. Influence of imazethapyr on ALS activity in (●) *Rhizobium leguminosarum* and (○) pea leaves. Vertical bars represent standard error of nine replicates.

This greater tolerance of *Rhizobium* ALS compared to that of plant origin is retained in the bacteroid state (Table 3). Thus, the plant fraction of nodules and the bacteroids not only showed higher ALS activities, but these were less sensitive than the ALS extracted from other plant organs (Table 3).

We conclude that *Rhizobium* tolerance to imazethapyr may be explained, at least in part, by a higher specific ALS activity and only marginally by a less sensitive target enzyme. Also, the greater tolerance to the herbicide by nodulated plants cannot be explained by a higher expression of ALS in leaves or roots. However, the plant fraction of nodules and, particularly, bacteroids do exhibit high ALS activities. The involvement of such enhanced nodular activities in the increased tolerance to imazethapyr deserves further investigation.

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